Microbial Profiling of Combat Wound Infection through Detection Microarray and Next-Generation Sequencing

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Combat wound healing and resolution are highly affected by the resident microbial flora. We therefore sought to achieve comprehensive detection of microbial populations in wounds using novel genomic technologies and bioinformatics analyses. We employed a microarray capable of detecting all sequenced pathogens for interrogation of 124 wound samples from extremity injuries in combat-injured U.S. service members. A subset of samples was also processed via next-generation sequencing and metagenomic analysis. Array analysis detected microbial targets in 51% of all wound samples, with *Acinetobacter baumannii* being the most frequently detected species. Multiple *Pseudomonas* species were also detected in tissue biopsy specimens. Detection of the *Acinetobacter* plasmid pRAY correlated significantly with wound failure, while detection of enteric-associated bacteria was associated significantly with successful healing. Whole-genome sequencing revealed broad microbial biodiversity between samples. The total wound bioburden did not associate significantly with wound outcome, although temporal shifts were observed over the course of treatment. Given that standard microbiological methods do not detect the full range of microbes in each wound, these data emphasize the importance of supplementation with molecular techniques for thorough characterization of wound-associated microbes. Future application of genomic protocols for assessing microbial content could allow application of specialized care through early and rapid identification and management of critical patterns in wound bioburden.

Modern combat environments create an array of difficulties relevant to the medical treatment of injured warfighters. Improvised explosive devices, increasing number and severity of injuries per casualty, and longer periods of time spent by the patient in transport represent unique challenges and necessitate a reassessment of our approach to wound management (1–3). The severity of blasts creates massive zones of injury that involve tissue, bone, and the neurovasculature. Since such wounds require serial debridements prior to definitive closure, surgeons must determine the optimal time for closure to reduce morbidity (4, 5). Although it has been shown that both infection and subsequent inflammatory pathology play an important role in wound progression, objective criteria for assessing and accurately estimating the likelihood of successful wound healing have yet to be clearly established (4, 6, 7).

Previous studies of wound infection have focused on a relatively small subset of well-characterized pathogens (8, 9). Recent studies have shown, however, that standard techniques overestimate the contribution of easily cultivated bacteria to the overall impact of the wound microbiota (10). Focusing only on cultured organisms reduces assessed diversity, and subsequent selection of single bacterial colonies applies a further bottleneck to downstream characterization. Analyses of chronic wounds have shown that the wound microflora is composed of a spatially structured (11–13) community of organisms that impacts healing either directly or indirectly through host immune and inflammatory responses (6, 14). Many of these organisms may be difficult or impossible to culture under

standard conditions, and their role in colonization of acutely wounded tissue is not well understood. In acute wounds, it is possible that organisms undetected by conventional techniques may impact the inflammatory response and play a significant role in the wound healing process.

Modern molecular techniques allow for comprehensive assessment of the microbial flora unique to each wound. These protocols provide superior reproducibility, precision, shorter assay duration, and lower overall costs for the acquired information compared to standard culture. These analyses could allow for personalized care based on the unique microbial flora of individual wounds rather than standardized treatment modalities directed toward a narrow range of microbes. These data could further be paired with assessment of the host inflammatory response to better estimate the likelihood of wound-spe-

cific complications. We believe that this type of comprehensive approach is needed to more completely understand the interacting roles of microbial communities and host response mechanisms in acute wounds. Samples obtained from the most complex of wounds, many of which are due to blasts, provide the ideal samples for these analyses.

We applied the Lawrence Livermore microbial detection array (LLMDA) for microbial analysis of 124 extremity wound samples (both tissue biopsy specimens and effluent from negative-pressure wound therapy [NPWT]) representing combat-injured U.S. service members. The LLMDA contains DNA probes capable of detecting all sequenced microbial species (15). This technology represents a cost-effective, high-throughput platform for analysis of wound infections, capable of detecting fastidious or unculturable organisms. We also employed whole-genome next-generation sequencing for high-resolution analysis of the complete wound microbiome in select samples. We applied a whole-genome methodology instead of targeted 16S rRNA gene sequencing, since a whole-genome approach facilitates deeper taxonomic resolution (16). Additionally, methods based on 16S rRNA genes will not identify plasmids or sequence derived from viruses or fungi, which could be relevant to the wound healing process. Although the unbiased whole-genome approach applied in this study may limit depth of individual species analysis, these methods are expected to more accurately represent bacterial abundance as indicated by sequence coverage.

By employing unbiased genomic technologies, we sought to examine microbial detection in the context of wound healing success or failure for identification of associations with clinical outcome. In combination with pathology and host response data, such information would be critically informative to combat wound management.

MATERIALS AND METHODS

Sample collection. Study participants were recruited from wounded U.S. service members evacuated from Iraq and Afghanistan to the National Capital Area. Samples were collected between September 2007 and January 2012. The study was approved by the Walter Reed National Military Medical Center Institutional Review Board (protocol number 352334, titled "The use of the vacuum assisted wound closure device in treating extremity wounds") in compliance with all federal regulations governing the protection of human subjects and informed consent. This study was a prospective, observational study and thus was exempt from registration.

Serial debridement of wounds was performed every 48 to 72 h with utilization of negative-pressure wound therapy (NPWT) application until surgical closure (4). Tissue and effluent samples were collected from 124 debridement procedures representing 61 wounds in 44 combat-injured patients. NPWT comprises attachment of a vacuum pump to the wound under an airtight, adhesive covering, which creates controlled negative pressure, causing effluent to flow from the wound bed into a sterile collection reservoir (without a gel pack; Kinetic Concepts, Inc., San Antonio, TX). A 1-cm^3 wound tissue specimen was obtained from the center of the wound bed at each debridement and divided, as described elsewhere (4, 6).

The tissue fraction for nucleic acid processing was immediately stored in RNAlater solution (Ambion, Austin, TX) at 4°C for 24 h. RNAlater was then removed, and the sample was stored at −80°C until analysis. The tissue fraction for quantitative bacteriology was placed in a sterile 15-ml conical vial and stored at 4°C until analysis, which typically occurred within 4 days. Samples were obtained from wounds that either healed

successfully or failed to heal. Wound failure was defined by reoperation for persistent drainage, progressive erythema, frank dehiscence following closure, or less than 90% engraftment of a split-thickness skin graft (4, 17). Each sample was assigned an identifier with three components: patient identification (ID) number, wound number, and sample type (e.g., 1-1-WA). Tissue biopsy specimens were designated "W," and effluent was designated "E." Samples were obtained from up to three procedures per wound (designated A, B, and C). The annotation "ON" indicates that an effluent sample was collected overnight starting the evening before debridement, while "2" indicates that the effluent was collected for 2 h following the procedure.

Quantitative bacterial culture from wound samples. Wound samples were examined for bacterial colonization using quantitative culture as previously described (6). Briefly, tissue biopsy specimens were fully homogenized with a sterile, disposable tissue grinder and diluted 1:10 (wt/vol) in fastidious broth (0.1 g tissue per ml). Dilute homogenates or wound effluents were inoculated (1 μl and 10 μl) on sheep's blood agar and MacConkey plates in triplicate prior to overnight incubation at 37°C. Following incubation, colonies were counted and the number of CFU per gram of tissue or ml of effluent was determined. Phenotypic identification of colonies was accomplished using the Phoenix automated bacterial identification system (Becton, Dickinson, Sparks, MD).

Nucleic acid processing. Wound tissue and effluent samples were homogenized and DNA was extracted using the QiaAMP DNA minikit and QIAamp cador pathogen minikit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Tissue samples of 15 to 20 mg were cut into small pieces and digested via incubation in proteinase K. Samples were disrupted via high-speed vortexing with 0.1-mm zirconia beads to facilitate disruption of Gram-positive bacteria. Purification of nucleic acid from sample homogenate was performed using QiaAMP spin columns. Fluorescent labeling of DNA was performed using the Nimblegen One-Color DNA labeling kit (Roche, Indianapolis, IN) according to the manufacturer's instructions (15).

Microarray processing and analysis. The LLMDA contains probes designed to detect all currently sequenced microbial pathogens. This study employed the 12-plex format of this array (version 5), which is restricted to pathogens associated with vertebrate infection, including 1,856 viral, 1,398 bacterial, 123 archaean, 48 fungal, and 94 protozoan species (current as of December 2011). Strategies for probe design and quality control have been previously published (15).

DNA samples were prepared for hybridization using the Nimblegen hybridization kit LS (Roche, Indianapolis, IN). Ten micrograms labeled DNA was hybridized to each array, followed by incubation for 45 to 60 h at 42°C. Arrays were washed using the Nimblegen wash buffer kit (Roche, Indianapolis, IN) and scanned using the Nimblegen MS200 microarray scanner. Microbial targets were identified as present or absent using composite likelihood maximization (CLiMax), a previously described web-integrated data analysis algorithm (15). Probe intensity thresholds were set at the 99th or 95th percentile of negative control probe intensities, as indicated.

Next-generation sequencing and analysis. Sequencing was performed by the Vincent J. Coates Genomics Sequencing Laboratory at the University of California, Berkeley. Overnight effluent samples (annotated E_ON) were prioritized for sequencing in order to maximize the quantity of bacterial sequence data relative to human background sequence. Samples were processed on the Illumina Hiseq 2000 platform using 100-bp paired-end reads, multiplexing three samples in each flow cell lane. Resultant data were processed using the Livermore Metagenomics Analysis Toolkit (LMAT), a software platform developed at Lawrence Livermore National Laboratory (LLNL) for scalable metagenomic taxonomy classification using a unique reference genome database (18).

Statistical analysis. Associations between microbial detection and wound healing outcome were assessed using Fisher's exact test. The Phyloseq package (version 1.6.0) in the R (version 3.0.2) software environment was applied for data interpretation and graphical representation

TABLE 1 Patient demographics and wound characteristics

Characteristic	Value for outcome	
	Healed	Failed
Total wounds	38	23
Age (yr), mean ± SD	23.2 ± 3.9	21.8 ± 2.3
No. of wounds		
Location		
Upper extremity	4	0
Lower extremity	34	23
Injury mechanism		
Blast	32	22
Crush	1	0
Gunshot	5	1
Injury severity score, mean ± SD	22.0 ± 13.3	25.2 ± 14.7
No. of wounds		
Wound type		
Soft tissue infection	19	14
Fasciotomy	9	6
Amputation	6	1
Open fracture	4	2
Closure type		
Primary	26	14
Integra	4	6
Graft	8	2
Flap	0	1

(19). Heat maps were built using h clustering in the gplots package (version 2.12.1) (20) or nonmetric multidimensional scaling (NMDS)-based ordering using a Phyloseq implementation of the NeatMap approach (21).

RESULTS

Patient demographics. Tissue biopsy and effluent samples were obtained from 124 debridement procedures representing 61 wounds in 44 patients with extremity war wounds. Wound locations, characteristics, and closure methods are given in Table 1. All patients were free of comorbid conditions, such as immune or connective tissue disorders, that could potentially confound results.

Microorganisms detected in wound samples by microarray. The LLMDA was applied for analysis of 124 wound samples, obtained from 44 patients. Microbial species detected in samples derived from each patient by both culture and LLMDA are shown in Table 2. A patient is indicated as positive for a given microorganism if any corresponding sample tested positive for that target. In cases where individual strains were detected, these were collapsed to the parent species. Complete microbial status and individual strain detection for all samples are shown in Data Set S1 in the supplemental material. At least one microbial target was detected by the LLMDA in 63 samples (51%). Wound samples were also interrogated for bacterial presence via quantitative culture. Of the 79 samples for which no bacterial growth was observed on culture, 27 (34% of culture-negative samples) were indicated to contain at least one microbial species. It was also observed in 22 samples (18% of total) that a species detected by culture was not identified by the corresponding microarray analysis. The most commonly observed microorganism in wound samples was *Acinetobacter baumannii*, represented in 28 samples (23% of total) (Fig. 1).

Association of microbial detection with wound resolution. Fifty-four of the 124 total samples (44%) were derived from wounds that failed to heal. Of the 61 total wounds examined, 22 (36%) failed to heal. Of the 44 patients from whom samples were derived, 16 individuals (36%) exhibited one or more failed wounds, with some patients demonstrating multiple wounds with different outcomes. LLMDA detection status for each individual sample is shown in Fig. 2, along with clinical outcome of the corresponding wound. Individual samples in Fig. 2 are clustered according to all the microbial targets identified by LLMDA in each sample. Samples derived from wounds with the same outcome did not cluster together in all cases, indicate multifactorial causes of wound healing failure. Table 3 shows wound failure rates segmented at the sample and wound levels. A wound was classified as positive for a microbial target if one or more derived samples tested positive. No association was observed between culture status and wound failure. When only effluent samples were considered, *A. baumannii* was detected by LLMDA in 14% of samples from healed wounds, compared to 27% for wounds that underwent dehiscence. Although this association with wound failure was not statistically significant ($P = 0.150$), a larger sample set could provide additional statistical power.

Multiple *Pseudomonas* species were detected in wound samples, including *P. aeruginosa*, *P. entomophila*, *P. putida*, and *P. stutzeri*. When considering effluent samples only, the LLMDA detected *Pseudomonas* in 3% of samples from wounds that healed successfully, compared to 17% of samples from wounds that failed to heal ($P = 0.059$). *Pseudomonas* were detected at the wound level in 3% of healed wounds and 23% of wounds that failed to heal ($P = 0.020$). Paradoxically, an inverse correlation was observed with detection of bacterial species associated with the gastrointestinal system (*Bacteroides fragilis*, *Bacteroides plebeius*, *Enterobacter cloacae*, *Enterobacter* sp., *Enterococcus faecium*, *Escherichia coli*, *Salmonella enterica*, and *Salmonella enterica* serovar Enteritidis). These organisms were detected most commonly in tissue samples, in which 30% of samples from healed wounds contained one or more of these species, compared to only 4% in those from failed wounds ($P = 0.013$).

In addition to the more commonly detected microorganisms, detection events in which species possibly relevant to wound healing were observed at low frequency also occurred. Examples included detection of three *Staphylococcus* species in one sample (*S. aureus*, *S. epidermidis*, and *S. lugdunensis*) and detection of the opportunistic pathogen *Mycobacterium abscessus*.

Association of *Acinetobacter* sequence-specific detection with wound resolution. One of the strengths of the microarray platform is the ability to detect sequence from plasmid DNA targets. The *Acinetobacter* plasmid pRAY was the most commonly detected plasmid sequence in wound samples. Within the effluent samples studied, only 3% of healed samples contained pRAY, compared to 23% of samples that underwent dehiscence ($P = 0.012$) (Table 3). In tissue samples, pRAY was detected in 24% of healed samples and 42% of failed wound samples ($P = 0.134$). When examining detection status by wound, pRAY was detected in 15% of healed wounds and 41% of failed wounds ($P = 0.029$).

Microbial diversity identified by next-generation sequencing. DNA extracted from a subset of 21 wound samples was subjected to whole-genome sequencing. All data were analyzed using the Livermore Metagenomics Analysis Toolkit (LMAT) (18). Due to elevated human content in these samples and our

TABLE 2 Microorganisms detected by culture and LLMDA for each patient[a]

Patient	Culture result	LLMDA detection[b]	Patient	Culture result	LLMDA detection[b]
1	*Pseudomonas stutzeri* *Citrobacter freundii*	*Mycobacterium abscessus** *Salmonella enterica** *Klebsiella pneumoniae** HPV71* *Acinetobacter* sp.	24	*Acinetobacter baumannii*	*Acinetobacter baumannii* Human parvovirus HPV71* *Salmonella enterica** *Borrelia afzelii** *Mycobacterium abscessus**
2	NG	None detected	25	NG	None detected
3	NG	*Acinetobacter* sp.	26	*Staphylococcus capitis* *Achromobacter* sp.	*Acinetobacter baumannii* *Pseudomonas putida* *Pseudomonas entomophila* *Ralstonia solanacearum** *Mycobacterium abscessus** *Borrelia afzelii**
4	NG	None detected			
5	NG	None detected			
6	NG	None detected			
7	NG	None detected			
8	NG	None detected			
9	*Acinetobacter baumannii*	*Acinetobacter baumannii* *Corynebacterium bovis* *Klebsiella pneumoniae**	27	*Acinetobacter* sp. Unidentified	*Acinetobacter baumannii* *Shigella boydii* *Shigella sonnei* *Escherichia coli** *Pseudomonas* sp.*
10	*Acinetobacter baumannii* *Enterococcus faecium*	None detected	28	NG	*Pseudomonas* sp.*
11	*Acinetobacter baumannii*	*Acinetobacter baumannii* *Salmonella enterica*	29	NG	None detected
12	*Acinetobacter baumannii*	*Acinetobacter* sp.	30	NG	None detected
13	NG	None detected	31	NG	*Bacteroides plebeius*
14	*Escherichia coli*	*Bacteroides fragilis** HPV57*			*Bacteroides fragilis* *Enterococcus faecium**
15	NG	HHV-6 *Pseudomonas* sp.* *Ralstonia solanacearum**	32	*Acinetobacter baumannii*	*Acinetobacter baumannii* HPV57
16	*Stenotrophomonas maltophilia* *Achromobacter* sp. *Moraxella* sp.	*Acinetobacter baumannii* *Achromobacter xylosoxidans* *Pseudomonas aeruginosa* *Staphylococcus epidermidis* *Staphylococcus lugdunensis* *Enterobacter cloacae* *Escherichia coli* *Bordetella avium* *Borrelia afzelii** *Staphylococcus aureus** *Klebsiella pneumoniae**	33	*Acinetobacter baumannii* *Achromobacter* sp.	*Acinetobacter baumannii* *Borrelia afzelii** HPV57*
			34	NG	None detected
			35	NG	None detected
			36	*Acinetobacter baumannii*	*Acinetobacter baumannii*
			37	NG	None detected
			38	NG	None detected
			39	ND	None detected
			40	*Acinetobacter baumannii*	*Acinetobacter baumannii* *Escherichia coli* *Klebsiella pneumoniae* *Salmonella enteritidis*
17	*Enterococcus faecium*	*Klebsiella pneumoniae** *Salmonella enterica** HPV57* HPV71*	41	NG	None detected
18	*Enterococcus faecium*	HPV57*	42	NG	None detected
19	*Bacillus cereus* *Acinetobacter* sp. *Alloiococcus otitidis* *Streptococcus agalactiae*	*Bacillus cereus* *Acinetobacter* sp. Human parvovirus *Pseudomonas stutzeri**	43	*Enterococcus faecium*	*Bacteroides fragilis* *Streptomyces avermitilis** HPV71* *Salmonella enterica** *Borrelia afzelii**
20	NG	None detected	44	*Acinetobacter baumannii* *Enterobacter cloacae*	*Acinetobacter baumannii* *Enterobacter cloacae* *Escherichia coli* *Klebsiella pneumoniae* *Salmonella enteritidis*
21	NG	*Roseburia hominis**			
22	NG	*Pasteurella multocida**			
23	*Acinetobacter baumannii* *Enterococcus faecium*	*Acinetobacter baumannii* Human parvovirus			Uncultured bacterium HPV71*

[a] Detection in all wounds and samples was collapsed at the patient level. Strain detection was collapsed at the species level. Complete detection data for all samples and strains are available in Data Set S2 in the supplemental material. NG, no growth; ND, culture not done; HPV, human papillomavirus.
[b] *, LLMDA detection at lower-intensity threshold (0.95).

whole-genome approach, the majority of total reads aligned to the human genome. Results revealed a broad range in mapped microbial sequence data between individual wound samples. Microbial sequence abundance was lowest for wound samples which tested negative both by culture and LLMDA (Fig. 3), suggesting that the extent of coverage for microbial sequence data derived from wounds is reflective of microbial status. No-

tably, the two samples with the highest microbial sequence abundance (26-1-ECON and 27-2-EBON) both tested negative for microbial presence via culture but positive by LLMDA. This may indicate that the relevant species successfully proliferated within the wound but, because of possible strain-specific factors, did not multiply under standard culture conditions and thus remained undetectable via standard microbiology. De-
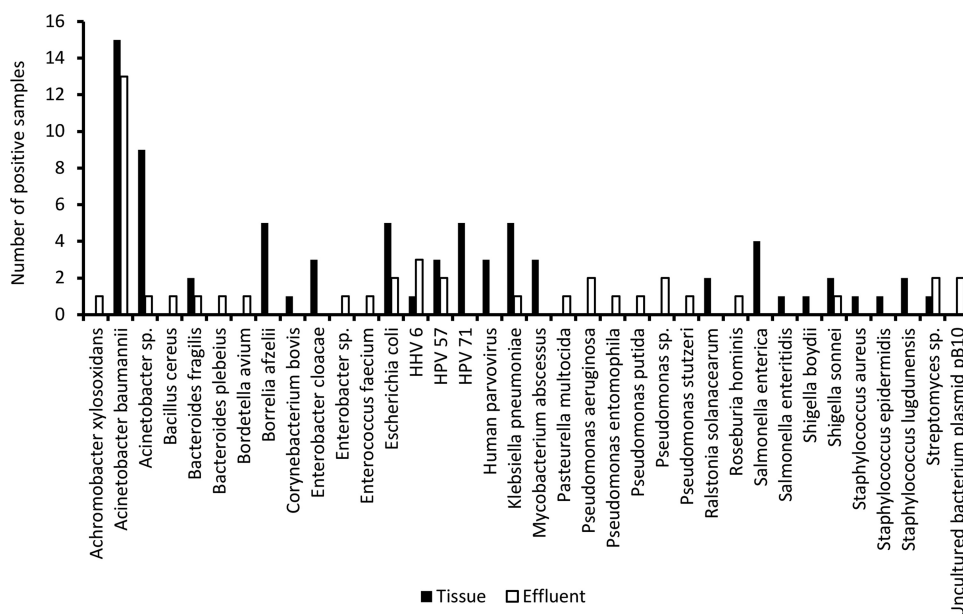
FIG 1 Microbial species detected by LLMDA in combat wound tissue and effluent samples. Nucleic acid was extracted from combat wound samples and hybridized to a microbial detection microarray. Tissue biopsy and effluent samples were analyzed independently. The number of positive detection events for each microbial target is shown. For samples in which a non-species-specific microbial target was detected (e.g., *Acinetobacter* plasmid) and no other species-specific target was observed in that sample, the sample was classified according to genus (e.g., *Acinetobacter* sp.).

tected bacterial species (via both LMAT and LLMDA) are shown for each sequenced sample in Data Set S2 in the supplemental material.

In the sample set examined, wound-derived samples did not group exclusively according to successful or failed healing based on microbial community composition. This is observed in Fig. 4A, in which samples were ordered according to their corresponding microbial profiles, and samples from wounds with similar outcomes were not observed to group together definitively (Fig. 4A). Figure 4B shows a heat map representing a subset of high-abundance microorganisms detected by LMAT analysis of sequence data. Again, samples did not group according to outcome. Further, samples derived from the same patient did not group as closely in this analysis as when all detected organisms were considered (Fig. 4A), indicating that the more comprehensive assessment of bioburden may yield a higher degree of clinical relevance. In one particular case in which multiple sampling time points were available (patient 26), samples obtained from the same wound at different times were observed to be compositionally distinct (Fig. 4C). In this wound, a population of *Acinetobacter* was present during the first procedure (sample EA2) and expanded during the following (sample EBON), while the latter procedures (samples EB2 and ECON) displayed prominent *Pseudomonas* detection. It should be noted that bacterial sequence coverage was higher in samples derived from sample ECON (Fig. 3), suggesting that colonization by *Pseudomonas* was characterized by greater bacterial abundance than *Acinetobacter*.

The relatively high sequence coverage for *A. baumannii* in some samples raised the possibility of identifying the strain associated with infection. Sequence alignment and unique single nucleotide polymorphism (SNP)-based identification approaches (22, 23) were therefore applied for strain resolution. These analyses revealed some clustering according to patient

and outcome; however, coverage and sample size were not sufficient to confidently assign a clinical correlation. These data and methods are detailed in the supplemental material (see Appendix S1).

Fungal and viral groups identified in wounds by next-generation sequencing. LMAT was also applied for examination of fungal and viral sequence data. For each identified fungal strain, total mapped reads and strain-specific mapped reads are shown, as well as the mean match score per read for each detected species/strain (Table 4). These scores were calculated as the ratio of the summed match score for all strain-specific mapped reads to the absolute number of strain-specific reads. Only those species with mean match scores exceeding 1.5, greater than 25 total mapped reads, and greater than 10 strain-specific mapped reads are shown. Reads corresponding to detected species were verified via BLAST analysis. Fungal species identified in wound samples above the set threshold included *Aspergillus terreus*, *Aspergillus nidulans*, *Alternaria arborescens*, *Saccharomyces cerevisiae*, *Malassezia restricta*, *Pseudoperonospora cubensis*, and *Neosartorya fischeri*. *Aspergillus* was detected in all samples derived from patient 26, whose wounds failed to heal. *Aspergillus* was also observed in one other sample derived from a failed wound and one from a healed wound.

Due to low observed microbial coverage and a relatively small genome size, viral identification could be reliably verified in only three samples. Identical filtering cutoffs were applied as described above (Table 4). *Pseudomonas* phage was observed in samples obtained from both wounds for patient 16, and both wounds failed to heal. This observation corresponded with the detection of multiple *Pseudomonas* species in these samples via sequencing and LLMDA. Human herpesvirus 6 (HHV-6) was detected in one sample derived from patient 15, in agreement with LLMDA detection.
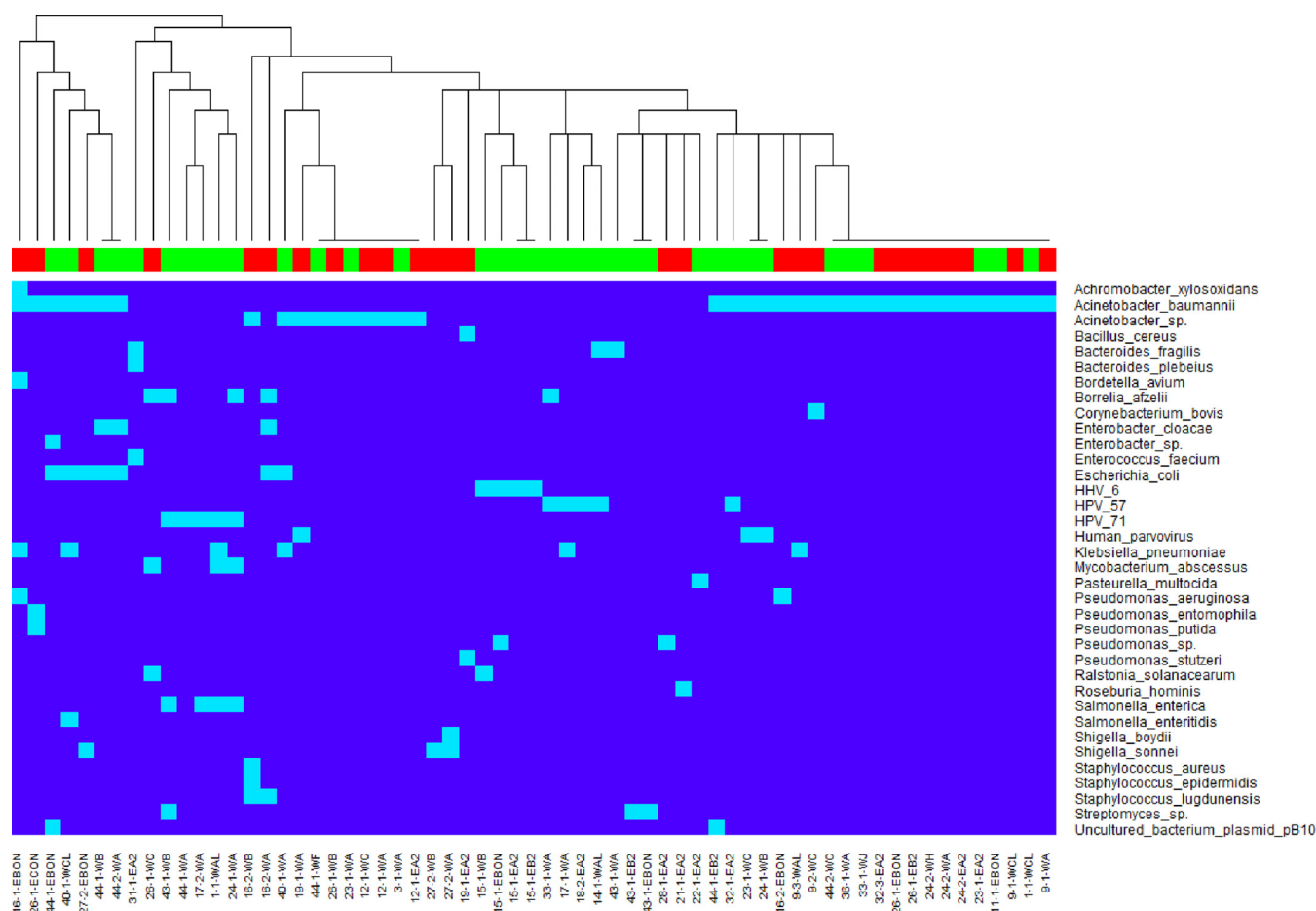
FIG 2 Clustering of samples from healed and failed combat wounds according to microbial species detected by microarray. Wound samples were ordered by hierarchical clustering. Samples were clustered according to their detected microbial profile, as determined by microarray detection. Individual patient samples are shown in columns and are labeled along the bottom horizontal axis. Patient samples are labeled according to the following scheme: patient number-wound number-extraction method (e.g., 9-1-WA). Sample extraction types are detailed in Materials and Methods. Detected microbial species are shown in rows and are labeled along the right vertical axis. As in the previous figure, when a non-species-specific microbial target was detected and no other species-specific target was observed in that sample, the sample was classified according to genus. Positive microbial detection is shown in light blue, and negative microbial detection is shown in dark blue. Wound outcome is indicated in a horizontal bar above the plot. Samples obtained from healed wounds are indicated in green, and samples from failed wounds are in red.

DISCUSSION

Studies of microbial colonization in traumatic wounds have been performed for combat injuries ranging from the Vietnam era (24) to modern environments (6, 25, 26) and in burn and chronic wounds from civilian populations (9, 10, 27). Although quantitative culture techniques were applied in this study, current standard practices for combat wound clinical microbiology involve only qualitative culture using surface swabs and phenotypic bacterial identification. These traditional culture methods result in an overestimation of the relative presence of easily cultured and identifiable microbes (10). Further, these techniques do not yield detailed genomic information, with the possible exception of drug resistance testing, the results of which can be slow and inconclusive.

This study shows that results from currently applied culture techniques underestimate the wound bioburden and do not correlate significantly with wound outcome. These methods should therefore be supplemented with molecular techniques. Microarray-based detection of *Acinetobacter* and *Pseudomonas* trended toward association with wound failure, although this correlation was not statistically significant at a *P* value of <0.05. It is likely, however, that a larger sample size would provide additional statistical power toward establishing this connection. This is supported by the fact that the *Acinetobacter* plasmid pRAY was significantly associated with wound failure. This plasmid has been implicated in multiple drug resistance, which may partially explain its association with wound failure (28). *Staphylococcus* was detected in only two wounds by microarray despite relatively frequent colonization of chronic and burn wounds by these bacteria. This observation may be owing to the unique conditions under which these wounds were acquired and treated, which are highly distinct from those of civilian injuries. It is therefore possible that incidence of this pathogen and amenability to colonization in these severe wounds are different from what might be observed elsewhere. The relatively low incidence of *Staphylococcus* is in agreement with previous studies of combat wound colonization (6).

One of the unique and statistically significant findings for these

TABLE 3 Correlation of target detection by microarray with wound resolution status[a]

Category	All samples			Individual wounds		
	No. (%) healed	No. (%) failed	P value	No. (%) healed	No. (%) failed	P value
All				39 (63.9)	22 (36.1)	
Effluent	37 (55.2)	30 (44.8)				
Tissue	33 (57.9)	24 (42.1)				
Culture				14 (35.9)	11 (50.0)	0.210
Effluent	7 (18.9)	8 (26.7)	0.321			
Tissue	16 (48.5)	11 (45.8)	0.528			
LLMDA				19 (48.7)	13 (59.1)	0.305
Effluent	13 (35.1)	12 (40.0)	0.437			
Tissue	23 (69.7)	15 (62.5)	0.386			
A. baumannii				10 (25.6)	9 (40.9)	0.171
Effluent	5 (13.5)	8 (26.7)	0.149			
Tissue	9 (27.3)	6 (25.0)	0.548			
Acinetobacter pRAY				6 (15.4)	9 (40.9)	0.029
Effluent	1 (2.7)	7 (23.3)	0.012			
Tissue	8 (24.2)	10 (41.7)	0.134			
Pseudomonas[b]				1 (2.6)	5 (22.7)	0.020
Effluent	1 (2.7)	5 (16.7)	0.059			
Tissue	0 (0.0)	0 (0.0)				
Enteric bacteria[c]				9 (23.1)	2 (9.1)	0.155
Effluent	2 (5.4)	1 (3.3)	0.579			
Tissue	10 (30.3)	1 (4.2)	0.013			

[a] Healing and failure rates are shown by individual sample and wound. Wounds were considered positive for a microbial target if one or more derived samples tested positive. All P values were calculated using Fisher's exact test.
[b] *Pseudomonas*: *P. aeruginosa*, *P. entomophila*, *P. putida*, *P. stutzeri*, and *Pseudomonas* sp.
[c] Enteric bacteria: *Bacteroides fragilis*, *Bacteroides plebeius*, *Enterobacter cloacae*, *Enterobacter* sp., *Enterococcus faecium*, *Escherichia coli*, *Salmonella enterica*, and *Salmonella enterica* serovar Enteritidis.

samples was the observation that enteric bacterial species associated more strongly with healed wounds. This seemingly paradoxical observation is in agreement with findings for chronic diabetic foot ulcers, in which ulcer duration correlated negatively with *Staphylococcus* abundance (29). This may reflect changes in immunoregulation and remodeling of the wound, where a microenvironment that is progressing toward healing is an amenable niche for distinct classes of bacteria from an environment that is immunologically distant from successful resolution. Detection of these microbial targets may provide a useful metric that is reflective of wound status. These results support the use of microarrays as a detection technology that could fill an important diagnostic niche, capable of delivering comprehensive microbial detection approaching the capacity of sequencing, but with monetary and time costs closer to those of PCR.

While microarray analysis revealed numerous instances where microbial presence was not detected by culture, cases in which a cultured organism was not detected by microarray analysis were also observed. Several factors may have contributed to this observation. Due to inherent differences in available sequence data between target organisms, probe coverage and detection capacity may differ between sequence targets, with the array exhibiting reduced power for identifying some organisms depending on reference genome quality and annotation. Addressing this issue is an active area of interest, and subsequent versions of the LLMDA are undergoing continuous

optimization. Also, while the Gram-positive bacteria *Enterococcus faecium* and *Alloiococcus otitidis* were detected by culture, *A. otitidis* was never identified via microarray and *E. faecium* was detected only once. While extensive bead beating was performed to facilitate Gram-positive lysis, it is possible that further optimization would improve extraction efficiency and detection capacity. Finally, the culture and molecular analyses were performed with different aliquots of tissue or effluent obtained from a given wound sample. It is possible that the microbes within these aliquots may not have been homogenously distributed. Discrepancies between culture-based and molecular techniques have similarly been observed in chronic wound studies, where results derived from distinct detection modalities depended on the bacterial group in question (30). These observations reinforce the difficulty in exclusive use of a single diagnostic tool and indicate that complementary approaches may be useful for yielding fully comprehensive results.

To further evaluate total wound bioburden, a subset of samples was selected for metagenomic analysis. Clear distinctions were not observed in overall community structure between successfully healed wounds and those that failed to heal. Failed wounds, however, did trend toward higher overall bacterial sequence coverage, and observations from one patient revealed that microbial status may shift over time. While the complete microbiome profile did not associate with clinical status, our observed microarray data
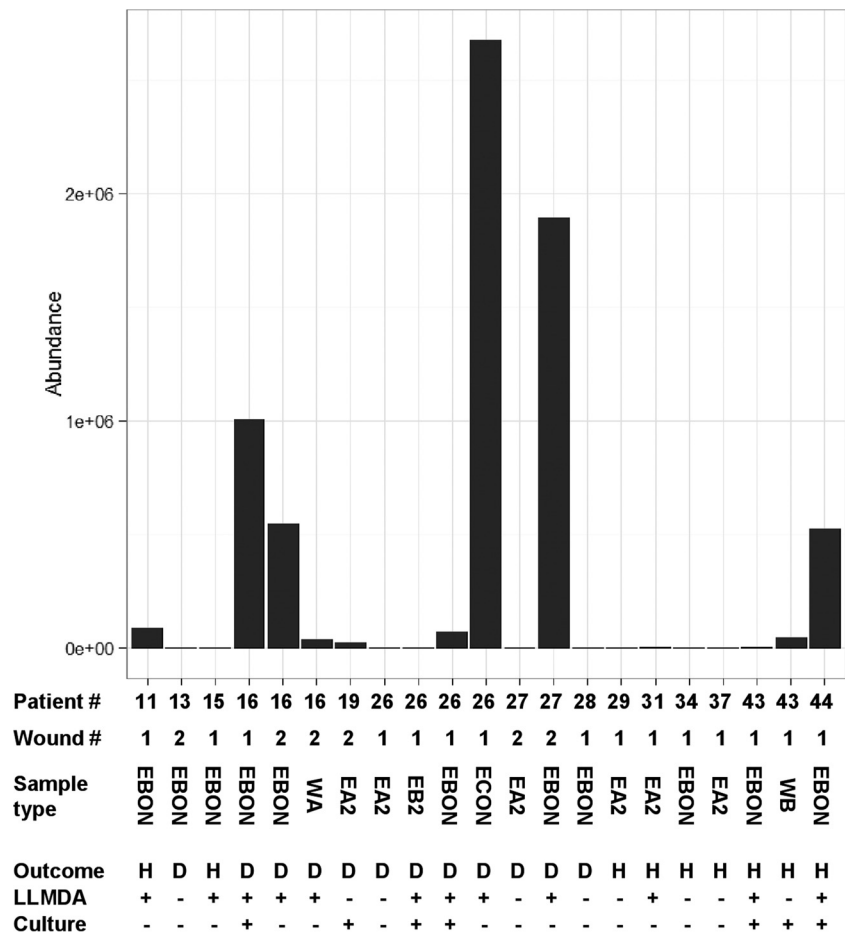
FIG 3 Quantity of next-generation sequence data mapped to microbial species by LMAT. Nucleic acid extracted from wound samples was processed via next-generation sequencing. Resultant reads were aligned to bacterial reference genomes using the Livermore Metagenomics Analysis Toolkit (LMAT). The abundance of reads mapped to microbial reference genomes is shown for each sample. Samples are shown along the horizontal axis, listed in numerical order by patient. The patient and wound from which each sample was derived are shown, along with sample extraction type (detailed in Materials and Methods). In some cases, multiple wounds from the same patient were analyzed. Metrics shown below the axis for each sample include wound clinical outcome (H, healed; D, dehiscence), LLMDA microbial detection status, and microbial culture status.

suggest that detection of specific microbial sequence targets does demonstrate association with wound outcome.

In some cases, sequencing analysis of wound samples revealed much larger numbers of taxonomic targets than were observed via LLMDA or identified organisms in samples with no targets detected by LLMDA. This was observed primarily in samples where less than approximately 2,000 reads were mapped to a given species/strain. This likely reflects the approximate limit of detection for the microarray platform, which is expected to be less sensitive than sequencing analysis (31). In other samples (e.g., 16-1-EBON), the total number of taxonomic targets was inflated by identification of many individual substrains, again due in part to the high sensitivity of sequencing, which could potentially have been collapsed in the sequencing analysis. It is likely that identification of these substrains is reflective of only a few actual colonizing strains.

In addition to species-specific detection, strain identity could also be relevant to wound care. This is particularly true in the case of *A. baumannii*, which is included in the ESKAPE pathogen group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*

and *Enterobacter* sp.), containing organisms especially capable of evading clinically applied microbicidal efforts (32). It has been shown previously that different species and strains within the *Acinetobacter* genus demonstrate distinct tolerances for pH and antimicrobial pressure (33). Further, separate *A. baumannii* strains derived from the same patient have been shown to be phenotypically distinct in a mouse model, as well as demonstrating different morphologies and propensities for catheter adherence (34). It has also been shown that differences in the infecting strain of *A. baumannii* can impact the resultant pathology and the cytokine response in a mouse model of pulmonary infection (35). The *A. baumannii* genotyping analysis outlined in Appendix S1 in the supplemental material did not attribute a specific response to the nearest-neighbor strains or a subset of unique SNP markers and thus was not a central focus of this study. However, these data do raise the possibility of classifying wound samples according to the most likely colonizing strain, and our application of alignment and SNP-based approaches demonstrates the potential of these tools for rapid genotyping of *A. baumannii*.

Although less common than bacterial colonization, fungal infections, in particular infection with *Aspergillus*, have been associ-
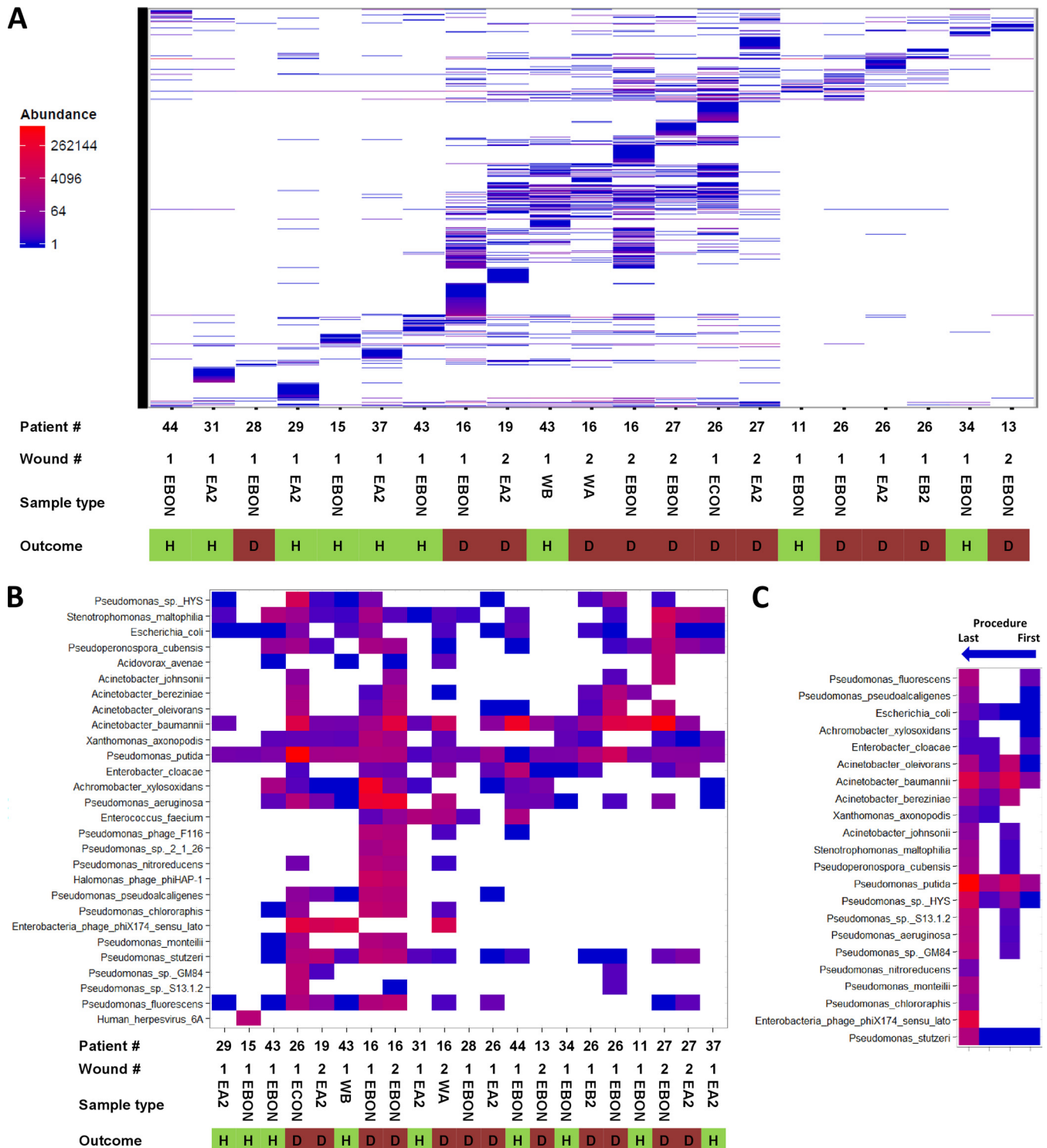
FIG 4 Microbial profiles in combat wound samples as determined by LMAT analysis of next-generation sequence data. Sequence data from wound samples were analyzed to determine the total quantity of reads mapping to each microbial species identified as present by LMAT. Microbial abundance within each sample, as measured by mapped reads, was used to order samples within a heat map using NMDS ordination by Phyloseq. Individual wound samples are given in columns. Labels below the horizontal axis show patient number, wound number, and sample extraction type (detailed in Materials and Methods). In some cases, more than one wound from the same patient was analyzed. Wound outcome is also indicated (H, healed; D, dehiscence). Microbial species are represented in rows and are shown along the vertical axis. (A) Heat map showing all microbial species detected by LMAT analysis. Individual species are not labeled due to the high number of total targets. (B) Heat map showing only those microbial species to which 1,000 or more total reads were assigned across all samples. (C) Heat map showing only those samples derived from patient 26, with samples ordered according to temporal collection point. Only those microbial species to which 1,000 or more total reads were mapped across all species are shown.

TABLE 4 Fungi and viruses identified in wound samples through analysis of next-generation sequence data with LMAT[a]

Sample	Wound outcome	Total no. of reads	No. of strain-specific reads	Mean match score	Species/strain
Fungi					
29-1-EA2	Healed	1,307	38	2.12	*Malassezia restricta* CBS 7877
43-1-EBON	Healed	27	25	2.65	*Aspergillus terreus* NIH2624
		76	60	2.31	*Aspergillus nidulans* FGSC A4
		27	19	2.28	*Alternaria arborescens* EGS 39–128
		1,193	74	1.65	*Pseudoperonospora cubensis*
43-1-WB	Healed	208	26	2.51	*Saccharomyces cerevisiae*
26-1-EA2	Dehiscence	145	134	2.26	*Aspergillus terreus* NIH2624
26-1-EBON	Dehiscence	35	29	2.26	*Aspergillus terreus* NIH2624
26-1-EB2	Dehiscence	70	68	2.40	*Aspergillus terreus* NIH2624
26-1-ECON	Dehiscence	39	34	2.33	*Aspergillus terreus* NIH2624
27-2-EA2	Dehiscence	2,281	37	2.75	*Aspergillus terreus* NIH2624
		118	47	1.91	*Pseudoperonospora cubensis*
27-2-EBON	Dehiscence	3,458	1,653	1.70	*Pseudoperonospora cubensis*
		35,693	36	1.68	*Neosartorya fischeri* NRRL 181
Viruses					
15-1-EBON	Healed	1,346	1,212	2.52	Human herpesvirus 6A
16-1-EBON	Dehiscence	2,219	843	1.72	*Pseudomonas* phage F116
		7,972	348	1.57	*Pseudomonas* phage phiCTX
16-2-EBON	Dehiscence	3,042	461	1.78	*Pseudomonas* phage phiCTX

[a] Fungi and viruses with more than 25 total mapped reads, more than 10 strain-specific mapped reads, and mean match scores exceeding 1.5 are shown.

ated with traumatic combat injury and can significantly complicate wound care (36, 37). One possible source of fungal material could be incorporation of contaminating organic matter at the time of injury (38). This is supported by detection of the fungal phytopathogens *Alternaria arborescens* and *Pseudoperonospora cubensis*, which may have been embedded concurrently with plant matter at the point of trauma, possibly consistent with the mechanism of injury from improvised explosive device (IED) blasts. Despite identification of fungal species via sequence data, fungi were not detected by the LLMDA, possibly due to minimal coverage or low quality and annotation of draft fungal reference sequences used for probe design.

Similarly, relatively few viral targets were identified with high confidence, and it is likely that elevated coverage would be required for reliable characterization. The majority of viral sequence data were expected to be derived from bacteriophage, and indeed, *Pseudomonas* phage was detected in two samples derived from separate wounds in the same patient. Further assessment of wound bacteriophage communities could be relevant to future development of novel phage therapy for addressing drug-resistant *A. baumannii* and *P. aeruginosa* infections (39, 40).

Wound failure is a consequence not only of the microbial bioburden but also of local and systemic inflammatory status. Numerous studies have identified human mediators of wound healing and the tissue remodeling response (6, 7, 17, 41). These studies have implicated a range of cytokines, chemokines, and matrix metalloproteinases, and support the notion that inflammatory dysregulation is central to wound healing failure. An increasing number of novel human biomarkers are being identified as predictive of wound healing progression (4, 42). The combination of molecular assays for host protein markers of inflammation with the advanced microbial detection protocols in this study could greatly improve care and reduce the high morbidity associated with blast and otherwise combat-related wounds.

In summary, these data support the inclusion of integrated molecular techniques for detection of microbial species and plasmid- or strain-specific sequences. Clinical assessment of the microbial flora unique to each patient could provide clinicians with invaluable information during the debridement process. More effective and timely assessments based on quantifiable metrics would reduce surgical morbidity, accelerate rehabilitation, and decrease the length of hospital stays. The potential for reduction in overall health care costs further supports the application of these molecular protocols as a prudent and cost-effective addition to the wound diagnostics armamentarium. These techniques could represent an important step toward personalized assessment of individual patients and rational design of tailored treatment regimens.

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